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In re Patent Application of:

Allan William CRIPPS, et al.

Application No.: 09/359,426

Filed: July 22, 1999

For: ANTIGEN

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Group Art Unit: 1614

Examiner: To Be Assigned

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Assistant Commissioner of Patents
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Sir:

Applicants are enclosing a certified copy of **Great Britain Patent Application No. 9701489.8** filed January 24, 1997. This document provides a basis for Applicants' claim for priority, which was made upon the filing of the above-identified patent application in the U.S. Patent and Trademark Office on July 22, 1999.

No fee is believed necessary with this submission. However, should the U.S. Patent and Trademark Office determine that additional fees are due upon the filing of this priority document, please charge any such fees to the undersigned's Deposit Account No. 02-0375.

Respectfully submitted,

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I also certify that the attached copy of the request for grant of a Patent (Form 1/77) bears an amendment, effected by this office, following a request by the applicant and agreed to by the Comptroller-General.

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The Patent Office

Cardiff Road
Newport
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1. Your reference

PWC/P19941GB

24 JAN 1997

2. Patent application number
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9701489.8

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

AUSPHARM INTERNATIONAL LIMITED
Level 2
220 St George's Terrace
Perth, Western Australia 6000
Australia

Patents ADP number *(if you know it)*

6627152001

If the applicant is a corporate body, give the country/state of its incorporation

AUSTRALIA

4. Title of the invention

ANTIGEN

5. Name of your agent *(if you have one)*

KILBURN & STRODE

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

30 John Street
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Patents ADP number *(if you know it)*

125001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature [signature] Date 24/January/1997

KILBURN & STRODE

12. Name and daytime telephone number of person to contact in the United Kingdom: Paul Chapman Tel: 0171 242 8291

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ANTIGEN

The present invention relates to a novel antigen from *Pseudomonas aeruginosa*, its use in medicine, particularly in the preparation of vaccines and in diagnosis.

P. aeruginosa is a Gram-negative aerobic motile bacterium with the form of rods. It is an environmentally ubiquitous, extracellular, opportunistic pathogen that causes significant morbidity and mortality in compromised subjects. Infection is of particular significance in subjects with cystic fibrosis, burns, chronic bronchitis, bronchiectasis and cancer.

Identification of immune responses, the search for vaccine candidates and suitable components for diagnostic tests have focused on components of *P. aeruginosa*. The outer membrane of *P. aeruginosa* contains toxins, including the lipopolysaccharide endotoxin, phospholipid and proteins. The various outer membrane proteins (Opr) of *P. aeruginosa* have been assigned an alphabetical naming system. While several proteins have been characterised by this scheme, the expression of some is only transient and highly dependent upon nutrient availability, culture conditions and the presence of antibiotics. Presently, three major Oprs, designated F,H2 and I, are recognised as antigenically common to and expressed in high copy numbers in all strains of *P. aeruginosa*.

We have now identified a protein from an outer membrane preparation of *P. aeruginosa*, which we have designated Pa60. The amino-terminal sequence of this protein does not demonstrate any sequence homology with other previously characterised proteins (GenBank data search). This protein is antigenic and is capable of inducing a protective immune response resulting in enhanced clearance of *P. aeruginosa*.

Thus, in a first aspect the present invention provides a protein antigen from *P. aeruginosa* and having a molecular weight in the range of about 60kDa to about 65kDa, as determined by SDS-PAGE.

In a preferred embodiment the protein has the following N-terminal sequence:

?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A; and preferably:

?-E-E-K-T-P-L-T-T- A- A- ?- A- P- V- V- ?- N- A.

Parts or fragments of the whole protein may themselves be antigenic and thus, in a second aspect, the present invention provides an antigenic fragment of the protein of the invention. In particular, the antigenic fragment will comprise the N-terminal sequence as described above.

The skilled man will appreciate that some variation in the sequence of fragments will be possible, while still retaining antigenic properties. Methods well known to the skilled man can be used to test fragments and/or variants thereof for antigenicity. Such variants also form part of the invention.

The antigenic protein, or fragments thereof, of the present invention can be provided alone, as a purified or isolated preparation, or as part of a mixture with other *P. aeruginosa* antigenic proteins.

In a third aspect, therefore, the invention provides an antigen composition comprising one or more proteins of the invention and/or one or more antigenic fragments thereof. Such a composition can be used for the detection and/or diagnosis of *P. aeruginosa*. In one embodiment the composition comprises one or more additional *P. aeruginosa* antigens.

In a fourth aspect, the present invention provides a method of detecting and/or diagnosing *P. aeruginosa* which comprises:

(a) bringing into contact an antigenic protein, or antigenic fragment thereof, or an antigen composition of the invention with a sample to be tested; and

(b) detecting the presence of antibodies to *P. aeruginosa*.

In particular, the proteins, antigenic fragment thereof or antigen composition of the invention can be used to detect IgG antibodies. Suitably, the sample to be tested will be a biological sample, e.g. a sample of blood or saliva.

In a fifth aspect, the invention provides the use of an antigenic protein, antigenic fragment thereof or antigenic composition of the present invention in detecting and/or diagnosing *P. aeruginosa*. Preferably, the detecting and/or diagnosing is carried out *in vitro*.

The antigenic protein, antigenic fragment thereof or antigen composition of the invention can be provided as part of a kit for use in *in vitro* detection and/or diagnosis of *P. aeruginosa*. Thus, in a sixth aspect, the present invention provides a kit for use in the detection and/or diagnosis of *P. aeruginosa* comprising an antigenic protein, antigenic fragment thereof or antigen composition of the invention.

In addition, the antigenic protein or antigenic fragment thereof of the invention can be used to induce an immune response against *P. aeruginosa*. Thus, in a further aspect, the present invention provides the use of an

antigen of the invention, a fragment thereof or an antigenic composition of the invention in medicine.

In yet a further aspect the present invention provides a composition capable of eliciting an immune response in a subject which comprises a protein or one or more antigenic fragments thereof of the invention. Suitably, the composition will be a vaccine composition, optionally comprising one or other suitable adjuvants. Such a vaccine composition may be either a prophylactic or therapeutic vaccine composition.

The vaccine compositions of the invention can include one or more adjuvants. Examples of adjuvants well known in the art include inorganic gels such as aluminium hydroxide or water-in-oil emulsions such as incomplete Freund's adjuvant. Other useful adjuvants will be well known to the skilled man.

In yet further aspects, the present invention provides:

(a) the use of a protein or one or more antigenic fragments thereof of the invention in the preparation of an immunogenic composition, preferably a vaccine;

(b) the use of such an immunogenic composition in inducing an immune response in a subject; and

(c) a method for the treatment or prophylaxis of *P. aeruginosa* infection in a subject, which comprises the step of administering to the subject an effective amount of a protein, at least one antigenic fragment or an antigen composition of the invention, preferably as a vaccine.

Preferred features of each aspect of the invention are as

for each other aspect *mutatis mutandis*.

The invention will now be described with reference to the following example which should not be construed as limiting the invention in any way.

The examples refer to the figure in which:

FIGURE 1: shows SDS-PAGE analysis of Pa60.

EXAMPLE 1: Protein Purification

Pseudomonas aeruginosa bacteria, strain 385 (Pa385), were harvested from overnight culture of 100 agar plates by scraping the plates followed by washing twice by centrifugation at 10,000 x g for 10min at 4°C. A crude outer membrane preparation was obtained by extraction of the outer membrane component with buffered Zwittergent 3-14 detergent and ethanol precipitation.

The outer membrane extract was lyophilised and resuspended in starting buffer (20mM Tris, pH8.5). This preparation was subjected to anion exchange chromatography using a Q2 column (BioRad) and a sodium chloride gradient to elute the proteins. The fractions eluted from the column were initially assessed for protein content by analytical SDS-PAGE. From this was determined the elution of profile for Pa60 allowing fractions containing Pa60 to be collected from susequent runs for further purification. These fractions were dialysed against distilled water, lyophilised, resuspended in a minimal amount of distilled water and further dissolved in 4 times the volume of sodium dodecyl sulphate (SDS) reducing buffer (62.5mM Tris, pH6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) ß-mercaptoethanol, 1.2×10^{-3}% (w/v) bromophenol blue). The SDS preparation

was incubated at 37°C for at least 30min prior to being loaded onto the stacking gel of the electrophoresis column.

Pa60 was purified using preparative polyacrylamide electrophoresis (PAGE). Preparative SDS-PAGE was performed using the BioRad model 491 Prep Cell using a 9% T-1.42% C acrylamide/BIS (N,N'-methylene-bisacrylamide) separating gel with a 10ml 4% T-0.36% C acrylamide/BIS stacking gel polymerised in a 28mm (internal diameter) column. Fractions eluted from the column were concentrated by lyophilisation and analysed for protein content by analytical SDS-PAGE. Pa60 isolated using these conditions contained SDS which was subsequently removed by potassium phosphate precipitation. fractions containing Pa60 were pooled and dialysed prior to determination of protein concentration.

Analytical identification of the protein was performed by analytical SDS-PAGE using either gradient 10-15% or homogenous 12.5% acrylamide gels and coomassie or silver stained. Protein concentration was determined using the Pierce micro BCA assay.

Results

Pa60 was successfully separated from other *P. aeruginosa* proteins by the described method. Figure 1 shows the position of this protein on SDS-PAGE.

EXAMPLE 2: N-terminal sequencing of Pa60

Pa60 was prepared for N-terminal amino acid analysis by excising the region containing the protein from an SDS-PAGE gel. The gel segments were sent to both the Biomolecular resouirce facility, Australian National University, Canberra, Australia and MUCAB Services, Macquarie University, North ryde, NSW, Australia.

Results

An N-terminal amino acid sequence was obtained which identified sixteen of the first nineteen amino acids. Possible amino acids were identified for the remaining residues and where there was uncertainty with a probable identification.

SEQUENCE:

	1	2	3	4	5	6	7	8	9	10
Definite		E	E	K			L			
Probable					T	P		T	T	A
Possible	S				A	L/S		A	I/D	W
	11	12	13	14	15	16	17	18	19	
Definite					V	V		N	A	
Probable	A		A	P						
Possible	F/L	G/S	N	D						

This provides a sequence with the following definite amino acids:

1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19

?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A

If one includes probable amino acids the following sequence is obtained:

1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19

?-E-E-K-T-P-L-T-T- A- A- ?- A- P- V- V- ?- N- A

EXAMPLE 3: Bacterial clearance following immunisation in a rat model

Specific pathogen free male rats received an intra-Peyer's patch (IPP) immunisation on day 1 and the live bacterial challenge on day 14. The animals were sedated by anaesthesia. The small intestine was exposed through a mid-line abdominal incision and the antigen injected subserosal to each Peyer's patch using a 27-gauge needle. The immunisation protein (Pa60) was prepared by emulsification of 200 or 800μg of protein per ml in a 1:1 ratio of Incomplete Freund's adjuvant (IFA) and phosphate

buffered saline (PBS) and a total inoculum of 10 or 40μg of protein respectively was administered to each animal. Animals were challenged for 4 hours with live bacteria (bacteria count 5 x 10^8 CFU) 14 days after the immunisation. Bacteria were grown overnight at 37°C in 5% CO_2 on nutrient agar plates, recovered, washed and resuspended in PBS to the required concentration. Bacteria were introduced into the lungs via an intra-tracheal cannula and 4 hours later the rats were euthanased. Blood was collected and aliquots of serum stored at -20°C for antibody analysis. Lungs were lavaged by flushing with 5 x 2ml of PBS and the pooled lavage (BAL) assessed for bacteria numbers. Following lung lavage, the lungs were removed, homogenised and assessed for numbers of bacteria. Cytospin slides were prepared for determination of differential cell counts in the lung lavage. total cell numbers present in the lung lavage were calculated by staining with trypan blue and counting using a haemocytometer.

Results

Rats immunised with Pa60 and challenged with live bacteria of the Pa385 homologous strain on day 14 showed an enhancement of bacterial clearance. Rats immunised with both 10μg or 40μg Pa60 had fewer bacteria recovered in both the BAL and lung than the non-immune group after 4 hours (Table 1).

Greater numbers of phagocytic cells were present in the BAL of Pa60-immunised animals and correlated with the enhanced bacterial clearance in these animals (table 2).

Table 1: Pulmonary clearance following Pa60 immunisation and challenge with *P. aeruginosa* (strain 385)

RAT GROUP	n[b]	*P. aeruginosa* recovered 4h post-challenge (log_{10}CFU)[a]	
		BAL	LUNG HOMOG.
NON-IMMUNE	5	7.63±0.11	8.66±0.18
10μg Pa60	6	6.95±0.07	8.43±0.09
40μg Pa60	4	7.19±0.07	8.37±0.19

Table 2: Cell count of Phagocytes in BAL following bacterial challenge

ANIMAL GROUP	TOTAL PHAGOCYTIC CELLS IN BAL
NON-IMMUNE	1.2 (±0.3) x 10^6
10μg Pa60	4.3 (±1.2) x 10^6
40μg Pa60	7.4 (±1.7) x 10^6

EXAMPLE 4: clinical diagnostic study

Children from the Royal Children's Hospital in Melbourne that had been diagnosed with cystic fibrosis provided samples for this study. Bronchoalveolar lavage (BAL) abnd serum were collected over a 3-4 year period from patients from the time of diagnosis as an infant. The samples were divided into groups based on clinical status of *P. aeruginosa*.

Group 1: Non-cystic fibrosis controls (age matched children with Stridor);

Group 2: Negative for *P. aeruginosa*;

Group 3: Upper respiratory tract isolation of *P. aeruginosa*, negative *P. aeruginosa* in lower respiratory tract;

Group 4: Cleared *P. aeruginosa* in the lower respiratory tract (negative in the next BAL sample); and
Group 5: Positive for *P. aeruginosa* in consecutive BAL samples.

An enzyme linked immunosorbent assay (ELISA) was used to measure antibodies to Pa60 in BAL and serum samples. Polysorb microtitre wells were coated with purified Pa60 at a concentration of 1μg per ml. The plates were washed five times in PBS containing 0.05% tween 20 between incubation steps. The wells were blocked with skim milk in PBS-0.05% Tween 20 for 60 min. Wells were incubated for 90 min with serum or BAL samples that were diluted in blocking buffer for analysis. Conjugated immunoglobulins used were rabbit anti-human IgG, IgA and IgM and wells were incubated with conjugated immunoglobulins for 90 min. The pltes were then developed. Human IgG, IgA and IgM were used to quantitate the antibody.

Results

An increase in antibody titre was observed as the incidence of infection with *P. aeruginosa* occurred. The non-cystic fibrosis control group and the non-infected cystic fibrosis patients had negligible titresto Pa60. Increased titres of IgG to Pa60 were observed, particularly in the patients with consecutive *P. aeruginosa* culture from the BAL (Group 5). In the BAL a significant increase in IgA titre was observed.

Table 3: Pa60-specific Antibody in Serum and Bronchoalveolar lavage from cystic fibrosis and non-cystic fibrosis children

PATIENTS	SERUM[a]			BAL[a]		
	IgG	IgA	IgM	IgG	IgA	IgM
GROUP 1	1.74	0.11	0.67	0.03	0.05	0.02
GROUP 2	1.40	2.34	2.10	0.03	0	0.02
GROUP 3	7.08 ±8.4	10.9 ±18	2.03 ±2.5	0.03 ±0.01	0.21 ±0.13	0.03 ±0.01
GROUP 4	18.9 ±21.9	0.56 ±0.6	1.46 ±2.07	0.02 ±0.01	0.12 ±0.04	0.01 ±0.01
GROUP 5	54.5 ±76	7.5 ±12.5	6.2 ±0.5	0.03 ±0.01	0.81 ±0.30	0.03 ±0.01

CLAIMS:

1. A protein antigen from *P. aeruginosa* and having a molecular weight in the range of about 60kDa to about 65kDa, as determined by SDS-PAGE.

2. A protein antigen as claimed in claim 1 which has the following N-terminal sequence:

?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A.

3. A protein antigen as claimed in claim 2 which has the following N-terminal sequence:

?-E-E-K-T-P-L-T-T- A- A- ?- A- P- V- V- ?- N- A.

4. An antigenic fragment of a protein as defined in any one of claims 1 to 3.

5. An antigenic fragment as claimed in claim 4 comprising the sequence:

?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A.

6. An antigenic fragment as claimed in claim 5 comprising the sequence:

7. An antigen composition comprising a protein as defined in any one of claims 1 to 3 or at least one antigenic fragment as defined in any one of claims 4 to 6.

8. An antigen composition as claimed in claim 7 which further comprises one or more other *P. aeruginosa* antigens.

9. A protein as defined in any one of claims 1 to 3, an

antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8 for use in the detection and/or diagnosis of *P. aeruginosa*.

10. A method of detecting and/or diagnosing *P. aeruginosa* which comprises:

(a) bringing into contact a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8 with a sample to be tested; and

(b) detecting the presence of antibodies to *P. aeruginosa*.

11. A method of diagnosing *P. aeruginosa* in a subject sufferring from cystic fibrosis which comprises:

(a) bringing into contact a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8 with a biological sample obtained from the subject; and

(b) detecting the presence of antibodies to *P. aeruginosa*.

12. A method as claimed in claim 10 or claim 11 wherein the sample is a sample of mucous, eg saliva.

13. The use of a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined

in claim 7 or claim 8 in detecting and /or diagnosing *P. aeruginosa*.

14. A method as claimed in any one of claims 10 to 12 or the use as claimed in claim 13 wherein the detecting and/or diagnosing is carried out *in vitro*.

15. A kit for use in the detection and/or diagnosis of *P. aeruginosa* comprising a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8.

16. A composition capable of eliciting an immune response in a subject which comprises a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8.

17. A composition as claimed in claim 16 which is a vaccine composition, optionally further comprising one or more adjuvants.

18. The use of a protein as defined in any one of claims 1 to 3, an antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8 in medicine.

19. The use of a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8 in the preparation of an immunogenic composition, preferably a vaccine.

20. The use of an immunogenic composition as defined in claim 19 in inducing an immune response in a subject.

21. A method for the treatment or prophylaxis of *P. aeruginosa* infection in a subject, which comprises the step of administering to the subject an effective amount of a protein as defined in any one of claims 1 to 3, at least one antigenic fragment as defined in any one of claims 4 to 6 or an antigen composition as defined in claim 7 or claim 8.

22. A method as claimed in claim 21 wherein the subject is sufferring from cystic fibrosis.

23. A method as claimed in claim 21 or claim 22 wherein the protein, one or more antigenic fragments or antigen composition is administered in the form of a vaccine.

24. A protein comprising the amino acid sequence:

?-E-E-K-?-?-L-?-?- ?- ?- ?- ?- ?- V- V- ?- N- A.

25. A protein comprising the amino acid sequence:

?-E-E-K-T-P-L-T-T- A- A- ?- A- P- V- V- ?- N- A.

Figure 1

Results

96
67
43
30
20
14